

# Developing a Marker of Exposure to Xenoestrogen Mixtures in Human Serum

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It has been hypothesized that environmental estrogens may play a role in the increasing incidence of breast cancer, testicular cancer, and other problems of the reproductive system. While a single causal agent can be identified in cases in which humans have had occupational exposures, wildlife showing signs of reproductive damage have usually been exposed to a combination of endocrine disruptors that may act cumulatively. The development of appropriate biomarkers of cumulative exposure, and their measurement at developmental points where exposure is critical, are required to test the environmental estrogen hypothesis. Measuring levels of each of the xenoestrogens in blood is a better approximation of real exposure at the target organ level than inferring cumulative exposure by estimating from mass balance of dietary levels. However, the cumulative estrogenicity of mixtures cannot be directly concluded from individual xenoestrogen plasma levels. Two approaches may be used to assess total load: *a*) the development of methods to study mixtures of these xenoestrogens, to quantify their cumulative effects, and to begin to understand their interactions (i.e., additivity, synergy, antagonism, or independent action), so that plasma concentrations may be translated into units of activity such as "estradiol equivalents"; and *b*) the development of methods to separate xenoestrogens from ovarian estrogens in blood and to directly measure the estrogenic activity of the xenoestrogen extract using a bioassay. The cumulative activity may be used as a marker of exposure to xenoestrogens. This article reports the development of a method to extract and separate xenoestrogens from ovarian estrogens using human serum as a source, followed by using a bioassay for determination of the cumulative xenoestrogen load as "estradiol equivalents." — *Environ Health Perspect* 105(Suppl 3):647–654 (1997)

Key words: xenoestrogens, estradiol equivalents, environmental exposure, *p,p'*-DDE, DDD, BBP, BPA, PCBs

## Introduction

Xenoestrogens include several lipophilic, persistent compounds to which humans and wildlife have been exposed. Among these synthetic chemicals are a number of chlorinated organics, such as the insecticides kepone, dieldrin, DDT and its metabolites, and some polychlorinated

biphenyl (PCB) congeners. More recently, nonchlorinated compounds used as antioxidants and plasticizers were found to be estrogenic (1–3). The detrimental effects of environmental exposure to xenoestrogens are evident when observing the abnormal development of the reproductive system in

gull embryos exposed *in ovo* to DDT and other pesticides (4). Similarly, the decreased reproductive success of alligators and turtles in Lake Apopka, Florida, was linked to a spill of kelthane, a pesticide formulation containing DDE (5). Other evidence that certain chemicals acted as endocrine disruptors was revealed when various occupational exposures in humans were reported. In 1949, aviation crop dusters handling DDT were found to have reduced sperm counts (6). While a single causal agent can be identified in cases in which humans have had occupational exposures, wildlife showing signs of reproductive damage are usually exposed to a combination of endocrine disruptors. Additionally, some of these chemicals may traverse the human placenta and have the potential to adversely affect the developing fetus (7). Although some of these effects may be obvious upon birth, others may not manifest themselves until sexual maturity has been reached. For example, boys exposed *in utero* to PCBs appeared normal during childhood, but at puberty their penises were significantly smaller than those of nonexposed controls (7).

It has been hypothesized that environmental estrogens may play a role in the declining quantity and quality of human semen during the last 50 years, as well as in the increased incidence of testicular cancer and cryptorchidism in males and breast cancer incidence in both females and males in the industrialized world (8–10). Regarding breast cancer, the main risk factor for its development is exposure to estrogens throughout an entire lifetime (11). Moreover, increased plasma levels of "bioavailable" ovarian estrogens (not bound to sex hormone-binding globulin [SHBG]) in postmenopausal women correlate positively with breast cancer (12).

Among the estrogenic xenobiotics, PCBs and DDT were considered suitable markers of exposure for breast cancer because they were released massively into the environment beginning approximately 50 years ago and they are persistent; their presence in serum may represent cumulative exposure during a lifetime. Three recent studies showed a correlation between the occurrence of breast cancer and levels of xenoestrogens. Wolff et al. (13) found that serum DDE levels correlated with breast cancer incidence in a study of 58 breast cancer patients and 171 controls that were well matched for risk factors and age.

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Abbreviations used: BBP, benzylbutylphthalate; BPA, bisphenol-A; CD, charcoal-dextran; CDHuS, CD human serum in phenol red-free DMEM; DDD, dichlorodiphenyldichloroethane; DDE, dichlorodiphenyltrichloroethylene; DDT, dichlorodiphenyltrichloroethane; DES, diethylstilbestrol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; E<sub>2</sub>, 17 $\beta$ -estradiol; EEQs, estradiol equivalents; HCB, hexachlorobiphenyl; HPLC, high-performance liquid chromatography; Pa, pascals; PCBs, polychlorinated biphenyls; PgR, progesterone receptor; RPE, relative proliferative effect; SHBG, sex hormone-binding globulin; tD, doubling time.

Another study documented that estrogen-receptor positive breast cancer correlated with higher concentrations of DDE in their tissues (14). Krieger et al. (15) studied 150 women with breast cancer and 150 controls; each set consisted of 50 African-American, 50 Caucasian, and 50 Asian-American women. When the data from all ethnic groups were pooled, no significant correlation was observed between plasma levels of DDE and breast cancer (15). However, when the cases and matching controls were evaluated separately, according to their ethnic group, high serum DDE levels were correlated with breast cancer incidence in Caucasian and African-American women but there was no significant correlation in Asian-American women. Evidence of a link between exposure to PCBs and breast cancer incidence is equivocal (16). However, these studies correlated exposure to total PCBs, rather than to the levels of specific congeners.

Epidemiological studies should be conducted involving larger sample sizes to clarify the relevance of these findings. It would be impractical to test only for the presence of DDT metabolites and PCBs since not all the PCB congeners are estrogenic, and many other environmental estrogens may also play a role. The newly identified estrogens may be less persistent than PCBs and DDT metabolites; however, they are widely used and exposure may occur steadily due to their presence in foods (3). Measuring the levels of each of the xenoestrogens in blood is a better approximation of real exposure at the target organ level than inferring cumulative exposure from estimation of mass balance of dietary levels. However, cumulative estrogenicity of mixtures cannot be directly deduced from individual xenoestrogen plasma levels. Two approaches may be used to assess total load: *a*) the development of methods to study mixtures of these xenoestrogens in order to quantify their cumulative effects and to begin to understand their interactions (i.e., additivity, synergy, antagonism, or independent action), so that plasma concentrations may be translated into units of activity such as "estradiol equivalents," and *b*) the development of methods to separate xenoestrogens from ovarian estrogens in blood and directly measure the estrogenic activity of the xenoestrogen extract using a bioassay. This cumulative activity may be used as a marker of exposure to xenoestrogens.

This article describes the extraction and separation of xenoestrogens from ovarian estrogens and phytoestrogens using human serum as a source. Next, methodologies to

determine the effect of xenoestrogen mixtures by means of the E-SCREEN bioassay are presented. This bioassay measures the proliferative effect of estrogens on their target cells. The proliferative effect of xenoestrogens is expressed as "estradiol equivalents."

## Materials and Methods

### Chemicals

Estradiol-17 $\beta$  (E<sub>2</sub>) was obtained from Calbiochem (Richmond, CA). DDT (technical grade), *o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDD, *p,p'*-DDD, *p,p'*-DDE, a PCB congener (2,2',3,3',6,6'-hexachlorobiphenyl [2,2',3,3',6,6'-HCB]), *o*-phenylphenol, *m*-phenylphenol, *p*-phenylphenol, and benzylbutylphthalate were from Ultra Scientific (North Kingstown, RI). Bisphenol-A was purchased from Aldrich Chemical Co. (Milwaukee, WI). Each of these chemicals was dissolved in ethanol or dimethyl sulfoxide (DMSO) before being tested in the various assay systems. The final solvent concentration in culture medium did not exceed 0.1%; this did not affect cell yields. The organic solvents, hexane, methanol, and 2-propanol were all of high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific (Pittsburgh, PA). Ethanol and ethyl ether were from Sigma Chemical Co. (St. Louis, MO) and petroleum ether was from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of the highest quality available from commercial sources.

### Plasma-derived and Blood-derived Human Serum

Plasma-derived human serum was prepared from outdated plasma supplied by the New England Medical Center Blood Bank. Calcium chloride was added to a final concentration of 30 mmol/liter to facilitate clot formation. Blood-derived serum was from blood samples obtained from healthy adult volunteers according to a protocol approved by the Human Investigation Review Committee at Tufts-New England Medical Center. After donation, blood was allowed to clot in glass centrifuge tubes for 2 to 4 hr to obtain serum. Plasma- and blood-derived sera were clarified by centrifugation (2000 $\times g$  for 10 min), stripped with charcoal-dextran (CD), and stored in glass tubes at -20°C until use.

### Removal of Sex Steroids by Charcoal-Dextran Treatment of Serum

Charcoal (Norit A, acid washed; Sigma) was washed twice with cold, sterile water

immediately before use. A 5% charcoal-0.5% dextran T70 (Pharmacia-LKB, Uppsala, Sweden) suspension was prepared. CD suspension aliquots of a volume similar to that of serum aliquots to be processed were centrifuged at 100 $\times g$  for 10 min. Supernatants were aspirated and serum aliquots were mixed with the charcoal pellets. This charcoal-serum mixture was maintained in suspension by rolling at 4 cycles/min at 37°C for 1 hr. This suspension was centrifuged at 2000 $\times g$  for 20 min. The supernatant was then filtered through a Nalgene filter with a pore size of 0.45  $\mu$ m. More than 99% of serum sex steroids were removed by this treatment, as measured by removal of <sup>3</sup>H-estradiol (17); estradiol concentrations after CD treatment were less than 0.01 pg/ml as measured by radioimmunoassay. CD-treated sera were stored at -20°C until needed. Samples kept in the freezer for 1 year maintained their inhibitory properties on the proliferation of human estrogen-sensitive breast tumor MCF-7 cells; plasma- and blood-derived sera were equally effective.

### Bioassay for Measuring Estrogenicity

Theoretical underpinnings of the E-SCREEN assay have been published elsewhere (17,18). Cloned MCF-7 cells were trypsinized and plated into 12-well plates (Falcon, Lincoln Park, NJ) at initial concentrations of 30,000 cells per well (19,20). Cells were allowed to attach for 24 hr; then the seeding medium (5% fetal bovine serum in Dulbecco's modified Eagle's medium [DMEM]) was replaced with the experimental medium [5% CD-treated human serum supplemented to phenol red-free DMEM (CDHuS)]. A range of concentrations of the test compounds was added to this medium. The bioassay was terminated on day 6 (late exponential phase) by removing the media from the wells, adding a cell-lysing solution (10% ethylhexadecyl-dimethylammonium bromide [Eastman Kodak Co., Rochester, NY] in 0.5% Triton X-100, 2 mM MgCl<sub>2</sub>, 15 mM NaCl, 5 mM phosphate buffer, pH 7.4) and counting the nuclei in a Model ZM Coulter Counter Apparatus (Coulter Electronics, Hialeah, FL).

The best estimate of the proliferative behavior of a cell population is doubling time (tD); the time interval in which an exponentially growing culture doubles its cell number. Determining tD requires measuring cell yields at several time intervals during the exponential proliferation phase. A less cumbersome alternative to measuring

proliferation rates is to compare the cell yield achieved by similar cell inocula harvested simultaneously during the late exponential phase of proliferation. The proliferative effect is measured as the ratio of the highest cell yield obtained with the test chemical to the hormone-free control. Under these experimental conditions, cell yield represents a reliable estimate of the relative proliferation rate achieved by similar inocula exposed to different proliferation regulators. In our experimental design, MCF-7 cell yields were measured 6 days after  $t_0$ ; however, significant differences between control and estrogen-treated cultures are already apparent after 4 days (21).

The estrogenic activity of xenobiotics was assessed by determining relative proliferative potency (RPP); this is  $100\times$  the ratio between the minimal concentration of estradiol needed for maximal cell yield at 6 days and the minimal dose of the test compound needed to achieve a similar effect, and by measuring relative proliferative effect (RPE); this is  $100\times$  the ratio between the highest cell yield obtained with the chemical and with estradiol. The RPE indicates whether or not the compound being tested induces a proliferative response quantitatively similar to the one obtained with  $E_2$ , that is, a full agonist ( $RPE = 100$ ), or a proliferative yield significantly lower than the one obtained with  $E_2$ , that is, a partial agonist. When combined with estradiol, antagonists significantly lower the proliferative yield obtained with estradiol alone.

### Antagonistic Effect

The E-SCREEN bioassay was also used to evaluate possible antagonistic activity of xenobiotics used in the cumulative effect experiments. Bisphenol-A (BPA) and  $p,p'$ -DDE were tested at a range of concentrations with and without the addition of 100 pM estradiol. The proliferative yield was compared to the steroid-free control. The differences between the estradiol-treated groups were assessed by a posteriori Shaffe's test (22). A  $p$  value of  $\leq 0.05$  was regarded as significant. Results were expressed as mean ratio of proliferative yield to steroid-free control  $\pm$  SE. Benzylbutylphthalate (BBP) and 2,2',3,3',6,6'-HCB were also tested using the same method.

### A Method to Determine Cumulative Effects of Xenoestrogens

The E-SCREEN bioassay was used to evaluate the cumulative effect of four xenobiotics: benzylbutylphthalate, bisphenol-A,

$p,p'$ -DDE, and 2,2',3,3',6,6'-HCB. Each of these xenoestrogens was tested for proliferative response at a range of concentrations from 10 pM to 100  $\mu$ M. From these dose-response curves, a minimal proliferative concentration was determined for each chemical. The xenobiotics, in three- and four-component mixtures, were combined at the minimal effective dose that produced a proliferative effect. Proliferative yield results, obtained from the mean cell number, were normalized to the steroid-free control to correct for differences in the initial plating density. These results were then converted to estradiol equivalents (EEqs), an activity unit representing xenoestrogen proliferative effect. The normalized mean cell number was used to interpolate EEqs from a normalized estradiol response curve. The minimal detectable EEq from the E-SCREEN bioassay was determined by  $t$ -test. A  $p$  value  $\leq 0.05$  was regarded as significant. The measured mixture EEqs were compared with calculated EEqs. Calculated EEqs for the mixtures were determined by adding the measured EEqs of the individual xenobiotics. The calculated EEqs for the mixtures represent the expected proliferative effect in the case of the three or four xenobiotic combinations interacting in an additive manner. Differences between the measured and calculated EEq values were assessed using chi-square analysis. A  $p$  value  $\leq 0.05$  was regarded as significant. The results were expressed as EEqs  $\pm$  SD. The cumulative effect experiments were conducted in duplicated wells and repeated 4 times.

### Extraction of Xenoestrogens from Plasma-derived Serum and Extract Cleanup

Extraction of xenoestrogens from serum was performed as described by Burse et al. (23), with modifications (1). To develop the extraction procedure, CD-serum was used for spiking and as a "blank" preparation. The extracts from the "blank" preparations are devoid of absorbance at 280 nm, and therefore do not interfere with the quantification of added xenoestrogens. In addition, CD-treated serum is lacking in estrogenic activity; this also holds for quantitation of the spiked estrogen by means of the E-SCREEN bioassay. To assess the recovery of xenoestrogens from serum, 4-ml aliquots of CD-treated serum were spiked with a known amount of standard xenoestrogen and allowed to equilibrate for 2 hr at room temperature.

**Sample Extraction.** Methanol (2 ml) was added to each of two 4-ml aliquots of

serum, mixed by vortexing, and then 5 ml of hexane: ethyl ether (1:1, by vol) was added to extract the mixture. The mixture was first agitated on a rotary mixer for 15 min and then centrifuged at  $2000\times g$  for 5 min. The organic phase was collected, and the aqueous phase was extracted twice more. The organic phases were pooled and subsequently concentrated to 1 ml by evaporation under a nitrogen stream.

**Acid Cleanup of the Organic Phase Prior to HPLC.** Concentrated sulfuric acid (0.5 ml) was added to the concentrated 1-ml sample of organic phase. The sample was briefly vortex-mixed and then centrifuged for 5 min at  $2000\times g$ . The organic phase was collected and the aqueous phase was extracted twice more with 1 ml of hexane. The organic phases were pooled and dried completely under nitrogen. The sample was resuspended in hexane and then injected into HPLC.

**HPLC Analysis/Sample Preparation for Measuring Estrogenic Activity by Bioassay.** Separation was performed by the method described by Medina and Sherman (24), modified by Sonnenschein et al. (1) in a Kratos chromatographer (solvent delivery system: Spectroflow 400; gradient controller and absorbance detector: Spectroflow 783; injector/mixer: Spectroflow 491; all from ABI Analytical, Inc, Santa Clara, CA). Aliquots of 500  $\mu$ l were injected into a  $4\times 220$  mm Spheri 5 silica column (Brownlee Labs, ABI) equilibrated with 100%  $n$ -hexane. Xenoestrogens were eluted by a gradient of two mobile phases:  $n$ -hexane (phase A), and  $n$ -hexane:methanol:2-propanol, (40:45:15 by vol) (phase B) at a flow rate of 1.5 ml/min and a pressure of  $5\times 10^{-6}$  pascals (Pa). The gradient was developed as follows: after injection, 100% phase A flowed for 2 min; then, the concentration of phase B increased from 0 to 400 ml/liter in 15 min and from 400 to 1000 ml/liter in the next 10 min. The elution profile was monitored at 280 nm with a Kratos Spectroflow 783 detector. All tested xenobiotics eluted during the first 10 min (1); they were evaporated completely and resuspended in ethanol. In the pilot experiment reported herein, the HPLC fractions from two 4-ml extracts were combined and resuspended in 140  $\mu$ l. This was done to allow for monitoring recoveries of spiked chemicals and proliferative activities in replicates. The efficiency of the step to solubilize xenoestrogens eluted from the HPLC column was tested by HPLC (percent recovery).

After these steps had been performed, 100  $\mu$ l of the remaining resuspended eluate

was pipetted into 9.9 ml of the CD-treated culture medium (5% CD-treated human serum). This sample is designated "undiluted preparation," and from it, the serial 2-fold dilutions were made; 1:1, 1:4, 1:8, 1:16, and 1:32. After equilibration at room temperature for 20 min, the preparation was rendered sterile by filtration through a 0.22  $\mu\text{m}$  (pore size) membrane. The dilutions were assayed by the E-SCREEN test. A "blank" was prepared from CD-treated human serum by following the fractionation protocol that was described for the preparations spiked with known amounts of xenoestrogens.

**Quantification.** To obtain calibration curves, chemicals to be analyzed were injected at five to seven different doses, ranging from 0 to 500 nmol. The correlation coefficient for each curve was 0.99.

## Results

### Identification of Xenoestrogens by the E-SCREEN Assay

The three isomers of phenylphenol, DDT (technical grade), and DDT metabolites were tested for estrogenicity (Table 1). Among the three isomers of phenylphenol, *p*-phenylphenol was a full agonist and the most potent (RPE = 88, RPP = 0.001); *o*-phenylphenol was the least potent and a partial agonist (RPE = 30, RPP = 0.0001). The meta isomer was also a partial agonist; its potency fell within the other two (RPE = 60, RPP = 0.0002).

*o,p'*-DDT, *o,p'*-DDD, and *p,p'*-DDT were full agonists; meanwhile *p,p'*-DDE, and *p,p'*-DDD were partial agonists. DDT (technical grade) was a full agonist; this was expected since it consists of 20% of *o,p'*-DDT and 80% of *p,p'*-DDT.

In testing for antagonistic effects, BPA (a full agonist) did not alter the proliferative effect of estradiol (Figure 1). BBP and 2,2',3,3',6,6'-HCB were also tested; they had no antagonistic effect (data not shown). The partial agonist *p,p'*-DDE showed statistically significant, partial antagonistic effects on MCF-7 cell proliferation (Figure 1).

### Development of a Method to Reveal Cumulative Effects of Xenoestrogens

The first step toward this end was to evaluate the sensitivity of the E-SCREEN test by means of a detailed estradiol dose-response curve (Figure 2). At any concentration below 1 pM (1 fmol in 1 ml culture medium), the mean cell number did not differ significantly from the steroid-free

**Table 1.** Estrogenic effect of industrial chemicals measured by the E-SCREEN assay.

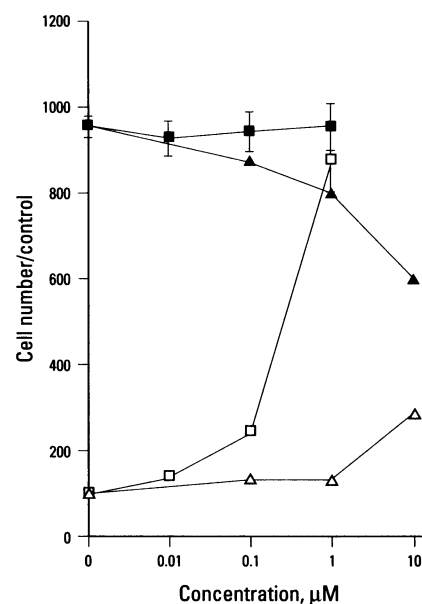
Compound	Concentration <sup>a</sup>	RPE, % <sup>b</sup>	RPP, % <sup>c</sup>
Estradiol	100 pM	100	100
4-Phenylphenol	10 $\mu\text{M}$	88	0.001
3-Phenylphenol	50 $\mu\text{M}$	60	0.0002
2-Phenylphenol	100 $\mu\text{M}$	30	0.0001
Benzylbutylphthalate	10 $\mu\text{M}$	90	0.001
Bisphenol-A	1 $\mu\text{M}$	82	0.01
2,2',3,3',6,6'-HCB	10 $\mu\text{M}$	62	0.001
DDT <sup>d</sup>	10 $\mu\text{M}$	80	0.001
<i>o,p'</i> -DDT	10 $\mu\text{M}$	86	0.001
<i>p,p'</i> -DDT	10 $\mu\text{M}$	71	0.001
<i>p,p'</i> -DDE	10 $\mu\text{M}$	25	0.001
<i>o,p'</i> -DDD	10 $\mu\text{M}$	73	0.001
<i>p,p'</i> -DDD	10 $\mu\text{M}$	19	0.001

<sup>a</sup>Indicates the lowest concentration needed for maximal cell yield. <sup>b</sup>Relative proliferative effect (RPE) is calculated as  $100 \times (\text{PE} - 1) / (\text{PE} - 1) \text{ of } E_2$ ; a value quantitatively similar to  $E_2$  (RPE = 100) indicates that the compound tested is a full agonist, a value of 0 indicates that the compound lacks estrogenicity at the doses tested, and a value significantly lower than  $E_2$  (RPE = 100) suggests that the xenobiotic is a partial agonist. <sup>c</sup>Relative proliferative potency (RPP) is the ratio between  $E_2$  and xenobiotic doses needed to produce maximal cell yields  $\times 100$ . All compounds designated as full or partial agonists (RPE > 15) increased cell yields significantly over the hormoneless control ( $p < 0.05$ ). <sup>d</sup>Technical grade.

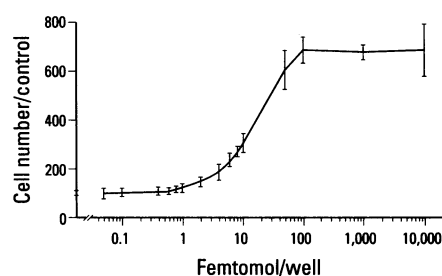
control ( $p \leq 0.05$  as determined by the *t* test). Therefore, 1 fmol of estradiol/well was determined as the lowest detectable estrogen amount in this assay. Mixtures of xenoestrogens were prepared to assess their cumulative effect. Each xenobiotic was set at a dose which would yield the minimal proliferative effect (the equivalent to 1 fmol estradiol). The xenobiotics BBP, 2,2',3,3',6,6'-HCB, BPA, and *p,p'*-DDE were combined in three and four component mixtures. Cell numbers were converted to estradiol equivalents by interpolation on the estradiol dose-response curve (Table 2) (measured cumulative effect). The effect of each xenoestrogen in the mixture was estimated; cell yields were converted to estradiol equivalents. These values were added to calculate the expected estrogenic effect of the mixture. Table 2 shows that the measured cumulative effects are significantly different from the expected values ( $p < 0.05$  as determined by chi-square analysis). This suggests that the cumulative effect of these xenoestrogens is synergistic rather than additive (Figure 3).

### Testing the Estrogenic Activity Extracted from Plasma-derived Serum

To measure the estrogenic effect of the spiked extracts, dose-response curves to



**Figure 1.** Antagonistic effects of *p,p'*-DDE and bisphenol-A. Cells were harvested after 6 days of exposure. Ordinate: cell number expressed as the treated/control ratio  $\times 100$ ; abscissa: concentration. Data points represent the mean of four experiments; error bars represent SE. Bisphenol-A was tested with (■) and without (□) 100 pM  $E_2$ . Shaffe's test showed no significant differences between the two treatment groups. *p,p'*-DDE was tested with (▲) and without (△) 100 pM  $E_2$ . Shaffe's test revealed significant differences between the two treatment groups ( $p < 0.05$ ).



**Figure 2.** Dose-response curve of estradiol. Cells were harvested after 6 days of exposure. Ordinate: cell number expressed as the treated/control ratio  $\times 100$ ; abscissa: concentration. Data points represent the mean of four experiments; error bars represent SD. *t*-test revealed significant differences between 1 fmol of  $E_2$  and the control ( $p < 0.05$ ).

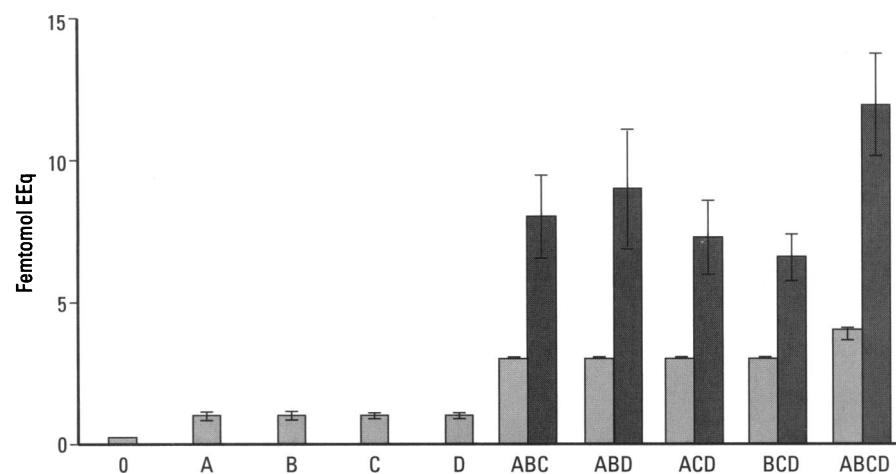
estradiol (Figure 4A) and *o,p'*-DDT (Figure 4B) were performed simultaneously with the E-SCREEN test of the extracts. The various dilutions of blank extracts obtained from CD-treated serum were assayed to assess whether toxic or spurious estrogenic activity was acquired during sample preparation. The cell yields were similar to those obtained when similar inocula were grown

**Table 2.** Cumulative effects of a mixture of four xenobiotics.

BBP, μM	BPA, μM	<i>p,p'</i> -DDE, μM	2,2',3,3',6,6'-HCB, μM	Mean cell #/ % control ± SD	Calculated EEq	Measured EEq
0	0	0	0	100 ± 10 <sup>a</sup>	—	0
2	0	0	0	127 ± 49	—	1.0
0	0.05	0	0	122 ± 18	—	1.0
0	0	2	0	125 ± 12	—	1.0
0	0	0	0.5	108 ± 10	—	1.0
2	0.05	2	0	271 ± 49	3.0	8.0
2	0.05	0	0.5	290 ± 68	3.0	9.0
2	0	2	0.5	256 ± 46	3.0	7.3
0	0.05	2	0.5	242 ± 30	3.0	6.6
2	0.05	2	0.5	329 ± 49	4.0	12.0

Cells were harvested after 5 days of exposure. BBP at 2 μM, BPA at 0.05 μM, *p,p'*-DDE at 2 μM, and 2,2',3,3',6,6'-HCB at 0.5 μM induce the minimal proliferative effect of 1 fmol of EEQs. EEQs of the xenobiotics were interpolated from the E<sub>2</sub> dose-response curve (Figure 2) using normalized mean cell number of four experiments ± SD. Calculated EEQs were determined by adding the EEQs of the mixture components. Measured EEQs were interpolated from E<sub>2</sub> dose-response curve (Figure 2) using the normalized mean cell number of four experiments ± SD. The calculated and measured EEQs for each mixture were significantly different using chi square analysis ( $p < 0.05$ ).

<sup>a</sup>Negative control: cell yield in 50 ml/liter CD-treated serum alone.



**Figure 3.** Cumulative effects of a mixture of four xenobiotics. Cells were harvested after 6 days of exposure. Data points represent the mean of four experiments; error bars represent SD. BBP (A) at 2 μM, BPA (B) at 0.05 μM, *p,p'*-DDE (C) at 2 μM, and 2,2',3,3',6,6'-HCB (D) at 0.5 μM induce the minimal proliferative effect of 1 fmol of EEQs. EEQs of the xenobiotics were interpolated from the E<sub>2</sub> dose-response curve (Figure 2) using the mean cell number of four experiments. Calculated EEQs (solid bar) were determined by adding the EEQs of the mixture components. Measured EEQs (shaded bar) were interpolated from E<sub>2</sub> dose-response curve (Figure 2) using the mean cell number of four experiments. The calculated and measured EEQs for each mixture were significantly different using chi square analysis ( $p < 0.05$ ).

in the presence of 5% CD-treated serum, indicating the absence of estrogenic activity (Figure 5A). Microscopic observation did not reveal any toxic effects. Additionally, a full estrogenic response was obtained when 100 pM estradiol was added to this preparation (Figure 5A). The spiked serum extract was also tested at various dilutions. The recovery of spiked *o,p'*-DDT was 84% (83 and 85%). Thus, the most concentrated dilution ("undiluted") was approximately 12 μM. As shown in Figure 5B, the dilution curve of the spiked preparation is in agreement with the dose-response curve to *o,p'*-DDT added directly to 5%

CD-treated human serum supplemented medium in Figure 4B. Lack of toxic activity in this extract is demonstrated by maximal estrogenic activity when each dilution of the spiked extract was tested in the presence of 100 pM estradiol (Figure 5B).

## Discussion

In addition to being exposed to their own steroidal estrogens, humans and wildlife are also exposed to synthetic xenoestrogens (pesticides, plasticizers, antioxidants, etc.) and natural ones, such as phytoestrogens. Exposure to synthetic estrogens, such as DDT, produces adverse effects in wildlife

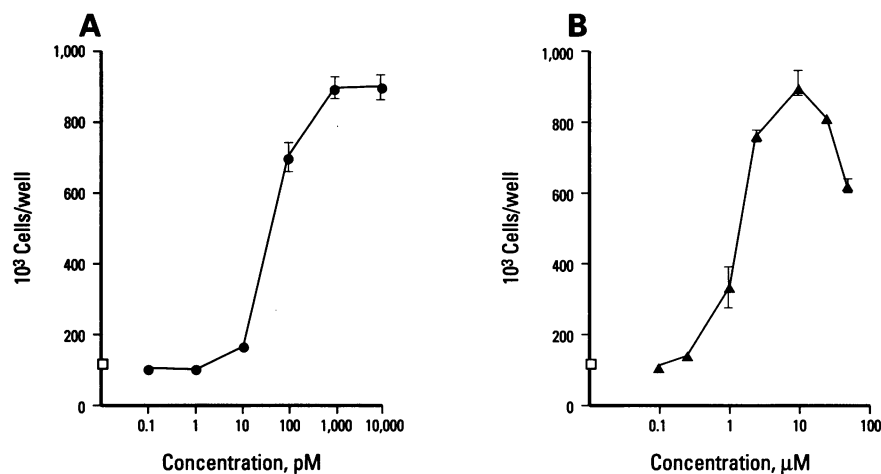
and humans. The lack of deleterious effects of phytoestrogens on human health may be explained by adaptive phenomena that developed slowly during the coevolution of plants and mammals. Sudden exposure of organisms to phytoestrogens that are foreign to their natural ecosystems may result in deleterious health effects (25). The role of the different estrogens (endogenous, synthetic, and phytoestrogens) in the etiology of cancer incidence and reproductive disorders awaits further, rigorous epidemiological studies that would correlate exposure to each type of estrogen with their subsequent health effects.

In this article, we evaluate the concept of measuring xenoestrogen exposure in humans by separating xenoestrogens from endogenous estrogens in human plasma or serum, and by measuring the combined estrogenicity of these xenoestrogens by means of a bioassay using estrogen-target cells in culture.

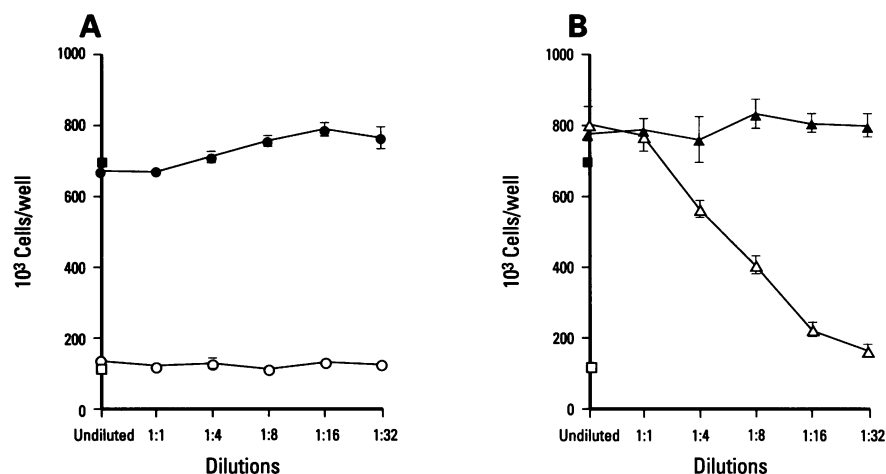
## Evaluation of Bioassays to Measure Estrogenic Activity

The hallmark of estrogen action is considered to be the ability of estrogens to induce proliferation of their target organs (26). Thus, a bioassay that measures cell proliferation as the end point is the most reliable assay to assess estrogenicity. The exposure of male birds, amphibians, and fish to nongonadal estrogens is well documented since the egg yolk protein vitellogenin is induced by estrogen in the liver, and its presence can be detected in blood. The presence of vitellogenin in the plasma of males or females whose reproductive tracts should be dormant is indicative of exposure. However, this marker cannot be used in reproductively active females since ovarian estrogens will induce vitellogenin and thus obscure the contribution of xenoestrogens to vitellogenin plasma levels. An equivalent marker of exposure has not yet been found in mammals.

Uterotropic assays measure increases of uterine wet weight, which are due to the additive effect of cell proliferation, hypertrophy, and water imbibition. While cell proliferation of the genital tract is specifically induced by estrogens, different end points are affected by hormones other than estrogens (3). Most estrogens produce similar effects in different species. However, tamoxifen is a full agonist in mice and a partial agonist and antagonist in rats and in human target cells (27). This may explain the discrepancy regarding the designation of some DDT metabolites as being estrogenic.



**Figure 4.** Dose-response curves of (A) estradiol and (B) *o,p'*-DDT on MCF-7 cells in culture. Cells were harvested after 6 days of exposure. Ordinate: cell number; abscissa: concentration. Data points represent the mean for a set of four experiments; error bars represent SD. (□) Negative control: cell yield in 50 ml/liter CD-treated serum alone.



**Figure 5.** Bioactivity of serum extracts on MCF-7 cell proliferation: dose-response curves of serial dilutions of (A) blank extract from CD-treated serum and (B) CD-treated serum spiked with *o,p'*-DDT. The ordinate represents cell yield after 6 days in culture. Data points represent the mean for a set of four experiments; error bars represent SD. (○) Blank extract. (●) Blank extract supplemented with 100 pmol/liter estradiol. (△) Extract from CD-serum spiked with 25 μmol/liter *o,p'*-DDT. (▲) Extract from CD-serum spiked with 25 μmol/liter *o,p'*-DDT supplemented with 100 pmol/liter estradiol. (□) Negative control: cell yield in 50 ml/liter CD-treated serum alone. (■) Positive control: cell yield in 50 ml/liter CD-treated serum plus 100 pmol/liter estradiol.

*p,p'*-DDE, the persistent, dominant metabolite of DDT, has been found to be a partial agonist by the E-SCREEN assay (3) (Table 1, Figure 1), while it is claimed by some to be devoid of estrogenicity in the rat model (28).

In addition to cell proliferation, other markers of estrogenic activity, such as the PgR assay and pS2 assay in MCF-7 cells, are adequate for assessing estrogenicity in humans. Cell proliferation assays are more sensitive than those that induce gene products (1,29–31). The transfection of the

estrogen receptor into yeast cells may create a vehicle in which to test various chemicals. It is expected that the use of reporter genes coding for an enzyme may result in a more sensitive and less time-consuming assay. However, the specificity and sensitivity of these methods have yet to be ascertained. In addition, the transport efficiency of xenobiotics may be different in yeast and human cells (32). Thus, the E-SCREEN assay appears to be the best candidate for establishing a quantitative standard of estrogenic activity in human cells at the

target-organ level, since no false positives or negatives have been observed among the estrogens and nonestrogens tested thus far.

### Novel Xenoestrogens Identified by the E-SCREEN Assay

Many chemicals that are present in our environment are estrogenic (19,33). Phenylphenol is one of the top 10 active ingredients in household pesticides and is the second most widely used ingredient for indoor applications (34). We reported previously that *p*-phenylphenol is a full estrogen agonist (20); *o*- and *m*-phenylphenol are partial agonists (Table 1). *o*-Phenylphenol is used in the rubber industry, in agricultural fungicides, and in disinfectants; *p*-phenylphenol is used in the rubber industry and in the manufacture of resins.

There has been controversy regarding which DDT metabolites are estrogenic, and also their relevance to breast cancer incidence (28). The E-SCREEN assay revealed that all the assayed metabolites are estrogenic. Additionally, full agonists are devoid of antagonistic effects (Figure 1); partial agonists are also partial antagonists (Figure 1). The antagonistic activity of these compounds, although statistically significant, is biologically marginal (Figure 1).

### Cumulative Effects

Environmental exposures are very different from most occupational exposures in which one chemical is found to be the causative agent. In environmental exposures, wildlife and humans are concurrently or sequentially exposed to a wide array of chemicals from various sources. Simultaneous exposure to mixtures may result in additivity, synergism, antagonism, or independent action. Toxicologists use a toxic equivalency factor model for dose conversion, a model based on the relative potency of the compounds to a standard compound (for example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the model or standard compound, and its congeners). This concept assumes that the potency of the two compounds is the same, regardless of the dose. Additionally, it assumes that the chemicals do not interact (synergism, antagonism). Using the E-SCREEN assay, mixtures of xenoestrogens may be tested, and the effects of mixtures compared to the calculated additive effect of each compound in the mixture (Table 2, Figure 3). The analysis of the interaction of xenoestrogens in the mixtures of three and four chemicals indicates that the effect of the mixture is 2- to 3-fold higher than the expected effect, assuming simple additivity.

This is of concern when deciding how to measure the cumulative exposure to estrogens in plasma, fat, or other biological samples. Measuring individual xenoestrogens and calculating cumulative exposure by deriving it from the relative estrogenic potency of each chemical may underestimate the estrogen load by ruling out synergistic effects. This problem may be obviated by directly measuring the effect of the mixture extracted from the biological specimen through a bioassay.

### A Method for Measuring the Cumulative Effect of Xenoestrogens in Human Serum

The extraction/clean-up methodology of pesticides from human serum, described by Burse et al. (23) and modified as described by Sonnenschein et al. (1), also allows quantitative extraction of more polar estrogens (from alkylphenols to estradiol). The method was further modified by eliminating the step that entailed passing the sample through a Sep Pak cartridge

(Waters Corporation, Milford, MA), since it results in coelution of unknown moieties somewhat toxic to MCF-7 cells (1). When the Sep Pak step was eliminated, the toxic effect disappeared (Figure 5). This method resulted in 83 to 85% recovery of the spiked *o,p'*-DDT. Moreover, the biological activity accurately paralleled the dose-response curve to this chemical. Therefore, it is now feasible to use this method to measure, in serum, the cumulative effect of the xenoestrogens tested thus far, since they were separated from ovarian estrogens.

We propose to measure the combined effect of the compounds eluted during the first 10 min of our HPLC separation as a marker of exposure to xenoestrogens. Estrogenic pesticides, PCBs, hydroxylated PCBs, phenolic antioxidants, and plasticizers elute from the HPLC column during the first 10 min, whereas ovarian estrogens, phytoestrogens, diethylstilbestrol, and mycoestrogens are retained for more than 12 min (1). Populations that will be investigated are not expected to have been

exposed to DES or mycoestrogens. Further optimization of the HPLC separation protocols may allow the separation of phytoestrogens from ovarian estrogens.

The data and analysis presented here represents an attempt to cope with intensifying demands by the public and regulatory agencies to rigorously evaluate the role of xenoestrogens in the increased incidence of male genital tract anomalies, testicular cancer, and breast cancer in human populations. In this article, we use novel extraction protocols and HPLC to measure the cumulative exposure to xenoestrogens. The full development of this concept into a reliable method to measure exposure in human populations will require further validation. Additional techniques, aimed at identifying all of the individual estrogenic xenobiotics present in serum samples of people suspected of being exposed, will help in correlating the relative contribution of each xenoestrogen with the observed health effect in exposed individuals.

### REFERENCES

1. Sonnenschein C, Soto AM, Fernandez MF, Olea N, Olea-Serrano MF, Ruiz-Lopez MD. Development of a marker of estrogenic exposure in human serum. *Clin Chem* 41:1888-1895 (1995).
2. Krishnan AV, Starhis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* 132:2279-2286 (1993).
3. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Olea-Serrano MF. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* 103:113-122 (1995).
4. Fry DM. Sex ratio skew and breeding patterns of gulls: demographic and toxicological considerations. *Stud Avian Biol* 10:26-43 (1987).
5. Guillette LJ, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ Health Perspect* 102:680-688 (1994).
6. Singer PL. Occupational oligospermia. *JAMA* 140:1249 (1949).
7. Yu M, Hsu C, Guo Y, Lai T, Chen S, Luo J. Disordered behavior in the early-borne Taiwan Yucheng children. *Chemosphere* 29:2413-2422 (1994).
8. Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm count and disorders of the male reproductive tract? *Lancet* 341:1392-1395 (1993).
9. Giwercman A, Carlsen E, Keiding N, Skakkebaek NE. Evidence for increasing incidence of abnormalities of the human testis: a review. *Environ Health Perspect* 101:65-71 (1993).
10. Davis D. Medical hypothesis: xenoestrogens as preventable causes of breast cancer. *Environ Health Perspect* 101:372-377 (1993).
11. Kelsey JL, Berkowitz GS. Breast cancer epidemiology. (Review) *Cancer Res* 48:5615-5623 (1988).
12. Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig KL, Shore RE, Strax P, Pasternack BS. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. *J Natl Cancer Inst* 87:190-197 (1995).
13. Wolff MS, Paolo G, Toniolo PG, Lee EW, Rivera M, Dubin N. Blood levels of organochlorine residues and risk of breast cancer. *J Natl Cancer Inst* 85:648-652 (1993).
14. Dewailly E, Dodin S, Verreault R, Ayotte P, Sauve L, Morin J, Brisson J. High organochlorine body burden in women with estrogen receptor positive breast cancer. *J Natl Cancer Inst* 86:232-234 (1994).
15. Krieger N, Wolff MS, Hiatt RA, Rivera M, Vogelstein J, Orentreich N. Breast cancer and serum organochlorines: a prospective study among white, black, and Asian women. *J Natl Cancer Inst* 86:589-599 (1994).
16. Wolff MS, Toniolo PG. Environmental organochlorine exposure as a potential etiologic factor in breast cancer. *Environ Health Perspect* 103:141-145 (1995).
17. Soto AM, Sonnenschein C. The role of estrogens on the proliferation of human breast tumor cells (MCF-7). *J Steroid Biochem* 23:87-94 (1985).
18. Soto AM, Sonnenschein C. Mechanism of estrogen action on cellular proliferation: evidence for indirect and negative control on cloned breast tumor cells. *Biochem Biophys Res Commun* 122:1097-1103 (1984).
19. Soto AM, Justicia H, Wray JW, Sonnenschein C. *p*-Nonylphenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environ Health Perspect* 92:167-173 (1991).
20. Soto AM, Lin T-M, Justicia H, Silvia RM, Sonnenschein C. An "in culture" bioassay to assess the estrogenicity of xenobiotics. In: *Chemically-Induced Alterations in Sexual Development: The Wildlife/Human Connection* (Colborn T, Clement C, eds). Princeton NJ:Princeton Scientific Publishing, 1992:295-309.
21. Soto AM, Silvia RM, Sonnenschein C. A plasma-borne specific inhibitor of the proliferation of human estrogen-sensitive breast

- tumor cells (estrocology-I). *J Steroid Biochem Mol Biol* 43:703–712 (1992).
22. Winer BJ. Design and analysis of single-factor experiments. In: *Statistical Principles in Experimental Design*. 2nd ed. New York:McGraw-Hill, 1962:149–260.
  23. Burse VW, Head SL, Korver MP, McClure PC, Donahue JF. Determination of selected organochlorine pesticides and polychlorinated biphenyls in human serum. *J Anal Toxicol* 14:137–142 (1990).
  24. Medina MB, Sherman JT. HPLC separation of anabolic oestrogens and ultraviolet detection of 17- $\beta$  oestradiol, zeranol, diethylstilboestrol or zearalenone in avian muscle tissue extract. *Food Addit Contam* 3:263–272 (1986).
  25. Shutt DA. The effects of plant estrogens on animal reproduction. *Endeavour* 35:110–113 (1976).
  26. Hertz R. The estrogen problem—retrospect and prospect. In: *Estrogens in the Environment. II: Influences on Development* (McLachlan JA, ed). New York:Elsevier, 1985;1–11.
  27. Martin L. Estrogens, antiestrogens and the regulation of cell proliferation in the female reproductive tract *in vivo*. In: *Estrogens in the Environment* (McLachlan JA, ed). New York:Elsevier/North-Holland, 1980;103–130.
  28. Safe S. Environmental and dietary estrogens and human health: is there a problem? *Environ Health Perspect* 103:346–351 (1995).
  29. Soto AM, Sonnenschein C. Cell proliferation of estrogen-sensitive cells: the case for negative control. *Endocr Rev* 8:44–52 (1987).
  30. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 47:4355–4360 (1987).
  31. Reiner GCA, Katzenellenbogen BS. Characterization of estrogen and progesterone receptors and the dissociated regulation of growth and progesterone receptor stimulation by estrogen in MDA-MB-134 human breast cancer cells. *Cancer Res* 46:1124–1131 (1986).
  32. Zysk JR, Johnson B, Ozenberger BA, Bingham B, Gorski J. Selective uptake of estrogenic compounds by *Saccharomyces cerevisiae*: a mechanism for antiestrogen resistance in yeast expressing the mammalian estrogen receptor. *Endocrinology* 136:1323–1326 (1995).
  33. Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen sensitive cells. *Environ Health Perspect* 102:380–383 (1994).
  34. Grossman J. Dangers of household pesticides. *Environ Health Perspect* 103:550–554 (1995).